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Short communication

Integrated protein analysis platform based on column switch recycling size exclusion chromatography, microenzymatic reactor and μRPLC–ESI-MS/MS

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ABSTRACT

An integrated platform with the combination of proteins and peptides separation was established via the unit of on-line proteins digestion, by which proteins were in sequence separated by column switch recycling size exclusion chromatography (csrSEC), on-line digested by an immobilized trypsin microreactor, trapped and desalted by two parallel C8 precolumns, separated by μRPLC with the linear gradient of organic modifier concentration, and identified by ESI-MS/MS. A 6-protein mixture, with Mr ranging from 10 kDa to 80 kDa, was used to evaluate the performance of the integrated platform, and all proteins were identified with sequence coverage over 5.67%. Our experimental results demonstrate that such an integrated platform is of advantages such as good time compatibility, high peak capacity, and facile automation, which might be a promising approach for proteome study.

1. Introduction

For proteome research, "bottom-up" approach has been regarded as one of the most popular analytical strategies. However, the simultaneous separation of thousands of peptides brings great challenges, not only to 2D-HPLC for peptides separation, but also to MS/MS for accurate proteins identification [1–4]. Therefore, proteins separation before digestion is of significance to decrease the complexity of samples prior to "bottom-up" based analysis. Recently, some approaches have been developed through off-line protein separation, digestion, peptide separation and protein identification [5,6]. However, by such methods, sample loss or contamination is unavoidable. Furthermore, it is difficult to achieve the automatic operation of whole systems. Therefore, the development of an integrated platform with combination of on-line protein separation, digestion, peptide separation and identification is imperative.

The key unit to integrate proteins and peptides separation is the rapid on-line protein digestion by immobilized enzymatic reactors (IMERs). In recent years, various IMERs have been developed [7–9], and the digestion time could be shortened even within 30 s [9]. Furthermore, IMER was also on-line coupled with HPLC for protein digestion and peptides separation. Liu et al. combined a gold nanoparticle assembly microfluidic reactor with 2D-HPLC/MS/MS for analyzing protein extracted from mouse morphages, and 497 proteins were identified within 20 h [10]. Schriemer and co-workers proposed an integrated platform to achieve protein separation by RPLC, on-line digestion by IMER, and protein identification by MS/MS [11,12]. However, to improve the biocompatibility of IMER with RPLC, the decreasing of organic modifier concentration in mobile phase by introducing another kind of buffer might also result in the dilution of samples. Furthermore, in the above-mentioned platforms, single-dimension separation could not offer sufficient resolving power and peak capacities for proteome analysis.

An ideal platform for complex protein sample analysis should be composed of on-line protein separation, digestion, peptide separation and protein identification. Furthermore, it was critical to keep good compatibility of each unit, no matter with the consideration of solvent polarity, flow rate, or operation time. Therefore, to meet the above-mentioned requirements, in our recent study, column switch recycling size exclusion chromatography (csrSEC), IMER and μRPLC–MS/MS was integrated, and successfully applied into the separation and identification of proteins mixture.

2. Experimental

2.1. Apparatus

MAGIC MS4 HPLC system was purchased from Michrom Bioresources (Auburn, CA, USA). Syringe pump was brought from Longer Precision Pump (Baoding, China). An LCQ^{Duo} electrospray ion trap

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mass spectrometer was ordered from Thermo Fisher Scientific (San Jose, CA, USA).

2.2. Chemicals and materials

Myoglobin (horse heart), transferrin (horse), ribonuclease (bovine pancreas), β-lactoglobulin (bovine milk), chicken egg albumin(ALB), carbonic anhydrase (bovine erythrocytes) and trifluoroacetic acid (TFA) were purchased from Sigma (St. Louis, MO, USA). Bovine serum albumin (BSA) was obtained from Shanghai Milk Company (Shanghai, China). Acetonitrile (ACN) of HPLC-grade was ordered from Merck (Darmstadt, Germany). Water was purified by a Milli-Q system (Millipore, MD, USA). All other chemicals and solvents were of analytical grade.

SEC *SRT*-150 Å-5 μm and SEC *nanofilm*-250 Å-5 μm particles were purchased from Sepax Technologies (Newark, Delaware, USA). XBP C18 particles (5 μm, 200 Å) were ordered from Bona Inc. (Tianjin, China). Acrylic polymer particles with epoxy groups (5 μm, 1000 Å) were donated by Shenzhen Nano-micro Technology Inc. (Shenzhen, China). C8 captrap precolumns (2 mm × 0.5 mm I.D.) were purchased from Michrom Bioresources.

2.3. Sample preparation

A 6-protein mixture (BSA, myoglobin, transferrin, carbonic anhydrase, ribonuclease and β-lactoglobulin) was denatured by 8 M urea, dissolving in 50 mM NH_4Ac buffer (pH 8.0), at 37 °C for 1 h, and then reduced with dithiothreitol at 56 °C. After being alkylated with iodoacetamide at room temperature, it was diluted with 50 mM NH_4Ac buffer until the total concentration of proteins reached 2 mg/mL.

2.4. Column packing

SEC *SRT*-150 Å-5 μm and SEC *nanofilm*-250 Å-5 μm particles were slurried in a mixture of water and alcohol (v/v = 2:1), and then packed into steel tubes (150 mm × 4.6 mm I.D. or 250 mm × 4.6 mm I.D.) under a constant pressure of 4000 psi.

C18 packing materials were slurried in bromoform, and packed into a Peeksil tube (50 mm × 0.3 mm I.D.) under a constant pressure of 6000 psi.

2.5. Preparation of trypsin immobilized microenzymatic reactor

Acrylic polymer particles with epoxy groups (about 40 mg) were added into 25% (v/v) NaOH solution, and then reacted at 40 °C for 3 h. After centrifuged at 1000 × *g* for 5 min, the supernatant was discarded, and the residual particles were packed into a steel tube (10 mm × 4.6 mm I.D.). After 5% (w/v) glutaraldehyde solution was continuously flushed into the column for 6 h using a syringe pump, 5 mg/mL trypsin dissolved in 50 mM borate buffer (pH 8.2) was pumped into the column overnight at room temperature, and trypsin was subsequently immobilized onto particles. Finally, trypsin immobilized microreactor was treated with 25 mM sodium cyanoborohydride overnight, and stored at 4 °C. Before usage, IMER was flushed with 10 mM ammonium acetate containing 50% (v/v) ACN to remove residual unbound trypsin.

2.6. SEC operation

SEC experiments were performed on a Magic MS4 HPLC system. Proteins were separated by a home-packed serially coupled SEC column (SEC *SRT*-150 Å and SEC *nanofilm*-250 Å). The mobile phase used for SEC was composed of 10 mM Tris–HCl, containing 150 mM NaCl and 5% (v/v) acetonitrile (pH 8.0). Proteins were eluted isocratically at a flow rate of 150 μL/min. Effluents were detected at 214 nm.

Recycling SEC (rSEC) experiments were performed on a home-set-up system, composed of an HPLC pump, two high-speed 10-port valves, two serially coupled SEC columns and a UV detector, based on which column switch recycling SEC (csrSEC), was developed. Fractions eluted from csrSEC were in sequence transferred into IMER for on-line digestion.

2.7. On-line protein digestion and peptides analysis by μRPLC–ESI-MS/MS

0.05 mg/mL BSA was pumped through IMER at 37 °C with the flow rate of 50 or 200 μL/min, and then the yielded peptides were collected, followed by the separation by a C18 column and identification by ESI/MS/MS.

The mobile phases for peptides separation was 2% (v/v) ACN containing 0.1% (v/v) formic acid (A), and 98% ACN containing 0.1% formic acid (B). The gradient was set as follows, 5% (0 min) → 5% (10 min) → 40% (70 min) → 80% (75 min) → 80% (80 min) B, at the flow rate of 5 μL/min. After each μRPLC separation, the column was equilibrated with the initial mobile phase.

2.8. Analysis of a 6-protein mixture by integrated platform

A 6-protein mixture (40 μg) was analyzed by our developed integrated platform, as shown in Fig. 1. To achieve good compromise between the resolution for proteins separation and time matching for peptides separation by μRPLC–ESI-MS/MS, proteins were

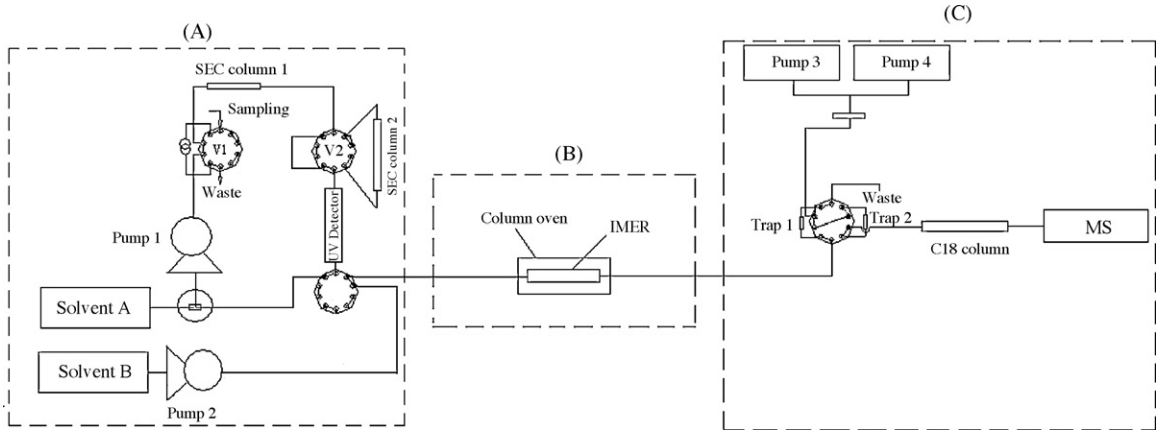

Fig. 1. Schematic diagram of integrated platform for protein analysis. (A) csrSEC; (B) IMER; (C) μRPLC–ESI/MS/MS.